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Positional Oxygen Isotope Exchange as a Probe for the Mechanism of Catalysis by *Escherichia coli* Succinyl Coenzyme A Synthetase†

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ABSTRACT: Succinyl-CoA synthetase of *Escherichia coli* has an $\alpha_2\beta_2$ subunit structure. The enzyme shows strict half-sites reactivity with respect to the phosphorylation of a histidine residue in the α subunit that represents a step in catalysis. Several lines of evidence indicate that this behavior may result from cooperative interactions between alternatingly functional active sites, so that subsequent steps in catalysis at one site may be promoted by phosphoryl transfer to the site on the neighboring half of the molecule. This study is directed toward learning more about the nature of these cooperative interactions. Here we have used positional isotope exchange (i.e., exchange of ^{18}O between the β,γ bridge and the β nonbridge position of ATP) as a test for transient bisphosphorylation. Succinyl-CoA synthetase was prepared in which one of the two active sites was thiophosphorylated; this species thus has one of its two active-site histidine residues occupied and unavailable for further reaction with ATP. Treatment of this monothiophosphorylated enzyme with [β,γ-^{18}O]ATP resulted in no significant scrambling of isotope into the nonbridge position, clearly indicating that the enzyme does not undergo even transient bisphosphorylation. We interpret the results in terms of a model of catalysis in which phosphoryl transfer to the second site occurs in concerted fashion with transfer from the first.

Succinyl-CoA synthetase catalyzes the substrate level phosphorylation step of the tricarboxylic acid cycle:

$$\text{succinyl-CoA} + \text{NDP} + P_i \rightleftarrows \text{succinate} + \text{NTP} + \text{CoASH} \quad (1)$$

The enzyme as isolated from *Escherichia coli*, the subject of this report, prefers adenine nucleotides as substrate and is of special interest because of the relationship of its quaternary structure to catalytic function. *E. coli* SCS[1] has been shown

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[1] Abbreviation: SCS, succinyl-CoA synthetase.

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to be a nondissociating $\alpha_2\beta_2$ tetramer with an overall molecular weight of 142000 (Bridger, 1974; Nishimura, 1986; Wolodko et al., 1986). The M_r values of the α and β subunits, calculated from their amino acid sequences, are 29600 and 41400, respectively (Buck et al., 1985). The overall reaction is believed to involve two covalent intermediates, namely a phosphoenzyme containing a phosphohistidine residue in the α subunit and an enzyme-bound succinyl phosphate. Three partial reactions (eq 2–4), in the direction of net synthesis of succinyl-CoA, may thus be visualized. An interesting property

$$E + MgATP \rightleftarrows E\text{–}P + MgADP \quad (2)$$

$$E\text{–}P + \text{succinate} \rightleftarrows E\text{·succinyl phosphate} \quad (3)$$

$$E\text{·succinyl phosphate} + CoASH \rightleftarrows E + \text{succinyl-CoA} + P_i \quad (4)$$

of the tetramer is that the enzyme displays strict half-sites reactivity with respect to phosphorylation—only one of the two apparently equivalent α subunits is phosphorylated by excess MgATP (Moffet et al., 1972).

A focus of research in this laboratory is to gain an understanding of the way in which the subunit structure of SCS contributes to its catalytic efficacy. A model has thus evolved, based upon a wide variety of experimental observations, that involves cooperative interactions between the $\alpha\beta$ halves of the molecule in the process of catalysis [see Wolodko et al. (1983) and Nishimura (1986) for detailed review and discussion]. The central feature of this model involves alternating cooperative effects between the two $\alpha\beta$ active sites, with interaction of ATP at one site causing, through conformational change, promotion of unfavorable catalytic events at the neighboring site. There is evidence that the formation of succinyl phosphate (reaction 3) may be the step that is promoted by these cooperative subunit interactions originating in the interaction of ATP at the neighboring site (Vogel & Bridger, 1982).

In the process of further refinement of this model for subunit interactions in catalysis, the foregoing considerations have raised the possibility that it is the phosphorylation of the second site, rather than simple binding in the Michaelis sense, that drives the proposed structural transition. In that case, despite the strict half-sites reactivity when the enzyme is tested with ATP, one could envision transient bisphosphorylation of the enzyme occurring before the release of product P_i from the first site. The simultaneous presence of two phosphoryl groups on one tetramer would not be detectable by ordinary sampling methods, since its lifetime would be less than one catalytic turnover. However, a more sensitive test for transient phosphorylation, positional oxygen exchange, has been introduced by Midelfort and Rose (1976) for the demonstration of transient phosphoryl transfer from donors such as ATP. This paper describes the application of this approach to succinyl-CoA synthetase.

Experimental Procedures

Enzyme. Succinyl-CoA synthetase was isolated from *E. coli* (Crooks strain) as described by Wolodko et al. (1986). The enzyme was stored at 4 °C as a suspension in 50% (w/v) ammonium sulfate solution, and solutions were dialyzed against the appropriate buffer before use. Protein concentrations were usually determined spectrophotometrically at 280 nm with a value of $E^{1\%}_{1cm}$ equal to 5.0 for *E. coli* SCS (Krebs & Bridger, 1974). When the presence of nucleotide precluded this, the method of Bradford (1976) was used, with pure SCS as standard. Enzymic activity was determined as described elsewhere (Bridger et al., 1969).

Thiophosphorylation of SCS. A 4 mg/mL solution of the enzyme in 0.05 M Tris-HCl, 0.05 M KCl, pH 7.4, was thiophosphorylated by treatment with [^{35}S]ATPγS as described previously (Wolodko et al., 1983). At timed intervals, 350-μL aliquots were removed and added to 150 μL of 0.2 M EDTA, pH 8.4, to stop the reaction. The enzyme was reisolated either by gel filtration on a 1 × 30 cm column of Sephadex G-25 equilibrated with 0.05 M Tris-HCl and 0.05 M KCl, pH, 7.4, or by centrifugation through 3-mL columns of Sephadex G-50 equilibrated with the same buffer, as described earlier (Wolodko et al., 1983). Samples of the labeled protein were removed for scintillation counting. The ultraviolet spectrum of this protein confirmed the absence of nucleotide.

Replacement of Thiophosphoryl Groups by Phosphoryls. SCS (40–60 μg) that had been either partially or fully thiophosphorylated as described above was then incubated with 20 μM [γ-^{32}P]ATP, 20 μM ADP, 1 mM $MgCl_2$, 0.05 M Tris-HCl, 0.05 M KCl, pH 7.4, in a final volume of 80 μL at 21 °C. After intervals of up to 10 min the reaction was stopped by the addition of 20 μL of 0.2 M EDTA, pH 8.4, and the entire sample was applied to a 1-mL column as described above, except that the Sephadex was topped with a 2-mm layer of Dowex 1-X8 (Cl form). The protein was isolated by centrifugation as described above, and the eluent was analyzed by liquid scintillation counting. In dual-labeling experiments, the specific activity of the [γ-^{32}P]ATP was adjusted so that the counts due to ^{32}P were significantly less than those from ^{35}S-labeled protein. The extent of overlap of ^{32}P into the lower energy ^{35}S window was subsequently determined by successive additions of ^{32}P internal standard to the samples. The effects of CoA and succinate on the relative proportions of –[^{32}P]PO_3 and –[^{35}S]PSO_2 (see Results and Discussion) were also determined by this method.

Synthesis of ^{18}O-Labeled ATP. Inorganic phosphate highly enriched in ^{18}O was synthesized by the method of Risley and Van Etten (1978) as modified by Hackney et al. (1980) and by O'Connor (1982). One gram of [^{18}O]H_2O (97.2 atom % ^{18}O) (Merck, Sharpe & Dohme Isotopes) was allowed to react with 1.7 g of PCl_5. The mixture was then neutralized by the addition of 2.4 g of imidazole, and the volume was made up to 200 mL with H_2O, followed by anion-exchange chromatography as described by Risley and Van Etten. Inorganic phosphate was detected by the method of Chen et al. (1956), and the isolated product was determined by ^{31}P NMR to consist of 92% $^{18}O_4$- and 8% $^{18}O_3$,$^{16}O_1$-labeled P_i. This labeled P_i was then converted to phosphoric acid by passage through a 1.5 × 6.5 cm column of Dowex 50-X8 (H^+), with elution by water. The eluent was concentrated by flash evaporation and used in the following synthesis. ATP labeled in each of the four terminal oxygens with ^{18}O was synthesized by reaction of ADP–morpholidate with the mono tri-*n*-butylamine salt of [^{18}O]P_i under anhydrous conditions for 42 h at 37 °C. The procedure described by Wehrli et al. (1965) was used, except that ion-exchange chromatographic purification of the product was carried out with use of DEAE-Sephacel (HCO_3^-). A typical yield of ATP was 65 μmol (32% based on ADP–morpholidate), and ^{31}P NMR showed the distribution of [$^{18}O_4$]ATP and [$^{18}O_3$,$^{16}O_1$]ATP to be 82% and 17%, respectively. The purity of the product was confirmed by thin-layer chromatography on polyethylenimine–cellulose using a 0.5 M LiCl/2 M formic acid solvent system (Mangold, 1965). After use in those experiments that resulted in no significant scrambling of oxygen (see Results and Discussion), [$^{18}O_4$]ATP was reisolated by chromatography on DEAE-Sephacel, with a linear gradient of 0–0.35 M triethylammonium bicarbonate

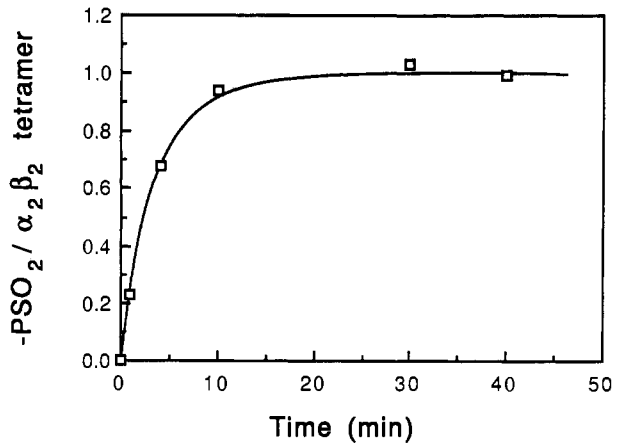


FIGURE 1: Thiophosphorylation of succinyl-CoA synthetase of *E. coli*. SCS (4 mg/mL) was incubated with [^{35}S]ATPγS as described previously (Wolodko et al., 1983). Aliquots were removed at 0.5, 3.5, and 15 min, and the partially thiophosphorylated enzyme was isolated as described in Experimental Procedures for use in subsequent experiments.

(pH 7.5). The labeled ATP was checked for purity and for ^{18}O distribution as described above and then reused for subsequent experiments.

Reaction of SCS with [$^{18}O_4$]ATP. The enzyme was thiophosphorylated (1.0 $-PSO_2/\alpha_2\beta_2$), and samples (60–100 μg of protein) were then incubated at 21 °C with 4 mM [$^{18}O_4$]ATP, 10 mM $MgCl_2$, 0.05 M Tris-HCl, 0.05 M KCl, pH 7.4, in a volume of 1 mL. Where indicated, certain samples also contained ADP, succinate, and/or CoA. After 0–10 min, the reaction was stopped by the addition of 0.5 mL of 0.2 M EDTA, pH 8.4. The protein was separated from nucleotide with the use of Amicon Centricon 10 microconcentrators that had been prewashed with 2 mL of the above Tris/KCl buffer containing 50 μg/mL of bovine serum albumin. The concentrators were centrifuged at 5000*g* for 60 min at 15 °C, and the ultrafiltrate containing the nucleotide at >99% recovery was stored at −20 °C for subsequent analysis by ^{31}P NMR. Protein recoveries, obtained by inverting the units and centrifuging according to the manufacturer's instructions, were >90%. Aliquots of these solutions were taken for protein determination (see above).

^{31}P NMR Spectroscopy. ^{31}P NMR was carried out with a Nicolet NW 300WB spectrometer at 121.46 MHz and 21 °C. ATP samples prepared as described above were diluted to 4 mL, resulting in final concentrations of 1 mM ATP, 35 mM EDTA, 0.05 M Tris-HCl, and 50% D_2O. The final pH was then adjusted to 9.10 ± 0.05 with small additions of KOH, and the samples were made to 4.0 mL. For each spectrum, 3000 scans were accumulated with a pulse angle of 70°, an acquisition time of 3.7 s, a sweep width of ±1100 Hz, and 16K data points. Zero filling to 32 K was applied prior to Fourier transformation, and spectra were recorded with an additional line broadening of 0.1 Hz. Peak areas were calculated by a curve approximation program and are expressed as percent of the spectrum for that particular signal.

Results and Discussion

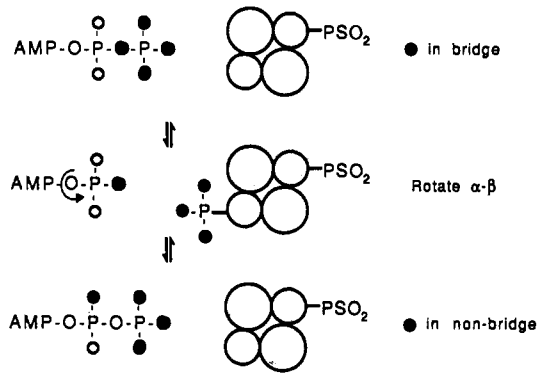
Thiophosphorylation of SCS and Displacement of $-PSO_2$ by $-PO_3$. Treatment of SCS with 0.5 mM [^{35}S]ATPγS produced fully thiophosphorylated enzyme (1 $-PSO_2/\alpha_2\beta_2$ tetramer) within 20 min at 21 °C (see Figure 1). Subsequent treatment of fully thiophosphorylated SCS with 20 μM [γ-^{32}P]MgATP and 20 μM MgADP (see Experimental Procedures) resulted in the gradual replacement of $-PSO_2$ by $-PO_3$, as shown in Figure 2. Significantly, at no time did the sum

Table I: Treatment of Monothiophosphorylated Succinyl-CoA Synthetase with Substrates: Effects on $-PSO_2$ and $-PO_3$ Content

conditions[a]	time (min)	$-PSO_2/\alpha_2\beta_2$	$-PO_3/\alpha_2\beta_2$
MgATP	0	0.94	0.00
MgATP	5	0.95	0.05
MgATP, MgADP	5	0.82	0.20
MgATP, MgADP, CoA, succ	5	0.74	0.02
MgATP (1 mM), CoA succ	2	0.17	nd[b]
MgADP	0	1.02	nd
MgADP	6	0.89	nd
MgADP, succ	6	1.02	nd

[a] ^{35}S thiophosphorylated enzyme (1 $-PSO_2$/tetramer) was incubated with substrates as shown. Unless otherwise indicated, the concentrations used were as follows: $MgCl_2$, 1 mM; [^{32}P]ATP, 20 μM; ADP, 20 μM; CoA, 10 μM; succinate, 1 mM. After the time interval shown, the reactions were stopped and the enzyme was isolated by centrifugation through gel filtration columns as described under Experimental Procedures. The contents of $-PSO_2$ and $-PSO_3$ in the enzyme were then estimated by liquid scintillation counting. All values are the means of duplicate determinations. [b] Not determined.

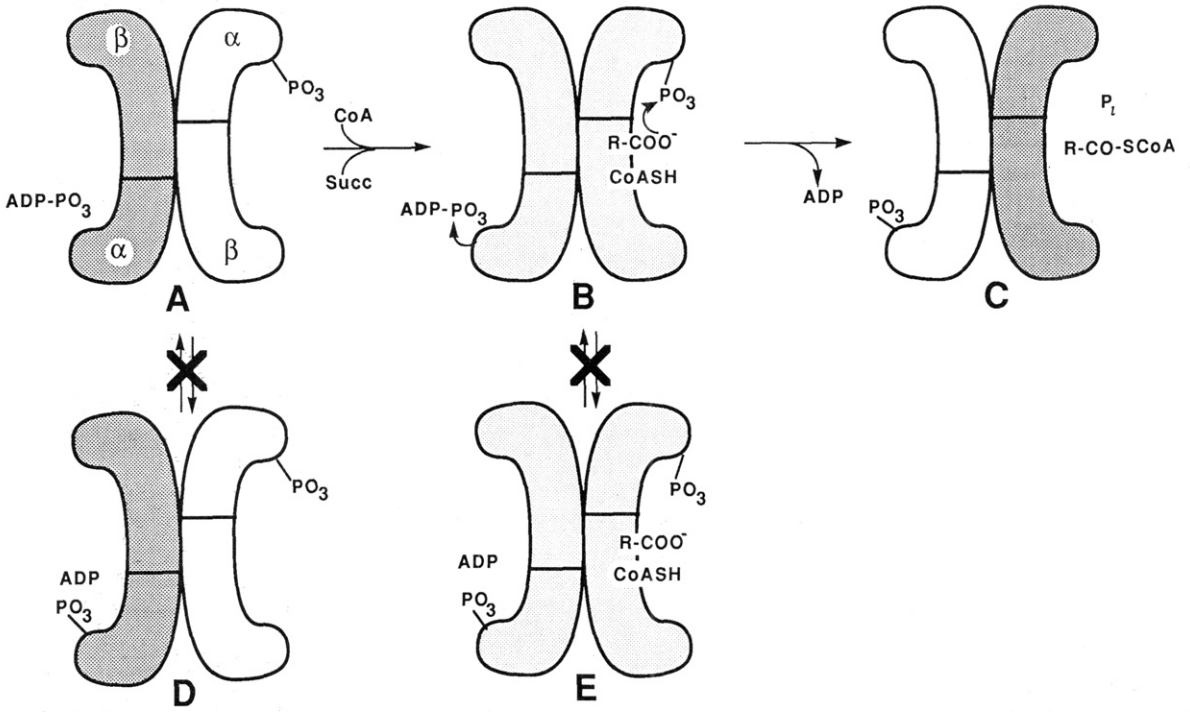
Scheme I: Positional Isotope Exchange as a Test for Transient Bisphosphorylation of Succinyl-CoA Synthetase (See Text for Details)



of $-PSO_2$ plus $-PO_3$ exceed one residue per tetramer, attesting to the rigorous half-sites behavior of this enzyme. Further, the data shown in Table I indicate that treatment of fully monothiophosphorylated SCS with MgATP, in the absence of other substrates, results in no removal of $-PSO_2$ nor incorporation of $-PO_3$. Addition of an equimolar mixture of MgATP and MgADP brings about a slow exchange of these groups. As expected, when MgATP is added together with succinate and CoA, almost complete displacement of $-PSO_2$ is observed, consistent with complete catalytic turnover. Taken together, these results are consistent with those reported previously (Wolodko et al., 1983) and are as we would expect if the $-PSO_2$ and $-PO_3$ groups were binding at the same site (i.e., the active site). The ability of MgADP to promote the displacement is the likely consequence of the reversal of the thiophosphorylation step (reaction 2 above), thus providing a significant steady-state level of the nonphosphorylated species for subsequent reaction with MgATP. Therefore, in an experiment in which fully monothiophosphorylated SCS is treated with [$^{18}O_4$]ATP in the absence of ADP, any scrambling of the β,γ bridge oxygen would have to be the result of transient phosphorylation at a second site, such as at the unoccupied active site in the neighboring half of the enzyme molecule. Similarly, scrambling that takes place only in the presence of MgADP could be attributed to reversal of reaction 2 above at a single site, without covalent reaction elsewhere. These considerations thus form the basis for the test for transient bisphosphorylation that we describe in the following section.

Positional Isotope Exchange. The rationale for this ex-

Scheme II: Model for Catalysis by *E. coli* Succinyl-CoA Synthetase[a]



[a] The unshaded and darkly shaded shapes represent $\alpha\beta$ halves of the tetrameric enzyme molecule with different conformations, which are shown to alternate in the course of catalysis. The lightly shaded shapes represent subunits with intermediate or transitory conformations. A–C: Proposed catalytic route, with phosphoryl transfer from ATP to left-hand site shown to be concerted with phosphoryl transfer from right-hand site to succinate. Further intermediate steps between the formation of succinyl phosphate and that of succinyl-CoA are omitted for clarity. The failure of the enzyme to catalyze positional isotope exchange in the absence of added succinate and CoA indicates that phosphoryl transfer at the second (left-hand) site does not occur separately or before phosphoryl transfer to succinate and that the A to D and B to E interconversions thus do not occur.

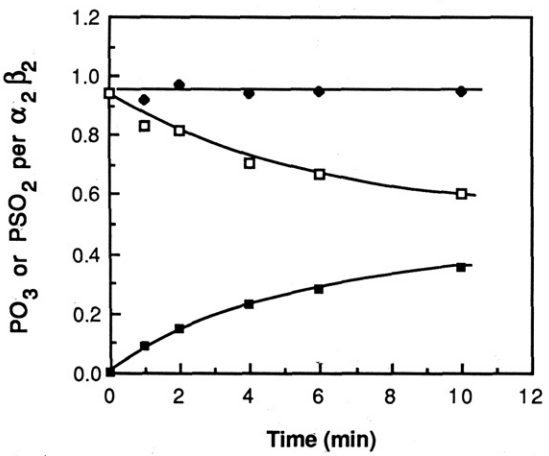


FIGURE 2: Replacement of thiophosphoryl groups of *E. coli* succinyl-CoA synthetase by phosphoryl groups. Fully thiophosphorylated ^{35}S-labeled SCS was incubated with 20 μM [γ-^{32}P]ATP and 20 μM ADP in the presence of 1 mM $MgCl_2$. Samples were removed at timed intervals, EDTA was added to 40 mM, and the enzyme was isolated by centrifugation as described in Experimental Procedures. The extent of ^{35}S thiophosphorylation (□) and ^{32}P phosphorylation (■) was determined by dual-labeling scintillation counting. The total of PSO_2 and PO_3 groups per tetramer is also indicated (♦). Similar results were obtained when partially thiophosphorylated enzyme (see Figure 1) was incubated with ATP and ADP as described above.

perimental approach is illustrated in Scheme I. Since one active site is effectively occupied by the presence of the nonexchangeable $-PSO_2$ group, positional isotope exchange can be used as a test for transient phosphorylation at the neighboring site. As shown in Scheme I, formation of a species in which the second site is phosphorylated, with dynamic reversal of the phosphorylation following simple rotation of the α,β bridge of ADP, would result in positional exchange of ^{18}O from the β,γ bridge ATP to the β nonbridge position.[2] Such exchange, taking place in the absence of other substrates, would carry the clear implication that the enzyme is capable of transient bisphosphorylation as a microscopic step in catalysis. The results of a test for positional isotope exchange are shown in Figure 3. The ^{31}P NMR spectra shown here are those of the doublet for the γ-phosphoryl group of ATP. Spectrum A is that of [$^{18}O_4$]ATP prepared as described under Experimental Procedures, showing the expected high proportion of species containing four ^{18}O atoms bonded to γ-P. Positional isotope exchange, with replacement of the β,γ bridge oxygen by ^{16}O, will increase the proportion of [$^{18}O_3$,$^{16}O_1$]P at the γ-phosphoryl, with an accompanying shift of 0.022 ppm (Cohn & Hu, 1980). Spectra B–D show, however, that no significant positional isotope exchange occurs when [$^{18}O_4$]ATP is incubated with monothiophosphorylated SCS, alone or together with either CoA or succinate. Only when CoA or succinate is added together with [$^{18}O_4$]ATP is there significant exchange, which is attributable to the ability of succinate and CoA to promote slow discharge of $-PSO_2$ from the enzyme (see Table I). Thus, the scrambling detected by spectrum E is the result of reversible monophosphorylation of enzyme that has been so freed of $-PSO_2$.

All of these results and those previously reported are consistent with the model for catalysis by succinyl-CoA synthetase that is shown in Scheme II. Here, the alternating conformers of the $\alpha\beta$ halves of the molecule are indicated by open and closed shading. Catalytic turnover must proceed via species A through C.[3] At B, phosphoryl transfer from ATP to the

[2] An implicit requirement in this and other applications of positional isotope exchange is that the transiently formed β-phosphoryl group of ADP is free to rotate fast enough to result in randomization of its three oxygen atoms.

[3] For clarity, intermediates containing enzyme-bound succinyl phosphate have been omitted between forms B and C.

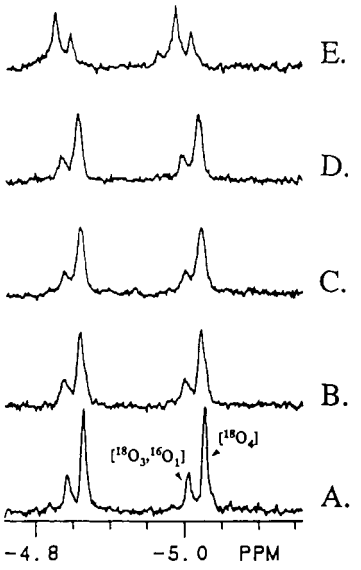


FIGURE 3: ^{31}P NMR spectra of the γ-phosphoryl of ^{18}O-labeled ATP, showing the distribution of $^{18}O_4$ and $^{18}O_3,^{16}O_1$ species. Samples containing 4 μmol of [$^{18}O_4$]ATP and 60 μg of monothiophosphorylated SCS in 1 mL of 50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, and 50 μM DTT, pH 7.4, were incubated at 21 °C for 0–6 min, with the additions as indicated below. The reaction was stopped by the addition of EDTA to 67 mM, and the nucleotide and enzyme were separated as described in the text. Each ultrafiltrate was made up to 4.0 mL as described to produce a final concentration of 1 mM [$^{18}O_4$]ATP, which was then analyzed by ^{31}P NMR. In spectrum A, EDTA was added prior to addition of enzyme and the ultrafiltrate was isolated as described above. Spectrum B shows the distribution of $^{18}O_4$ and $^{18}O_3,^{16}O_1$ species present after 6 min of incubation with the enzyme. Spectrum C: Conditions as in B but also containing 50 μM CoA in the incubation mixture. Spectrum D: As in B but also containing 10 mM succinate. Spectrum E: As in B but containing both 50 μM CoA and 10 mM succinate in the incubation mixture. The stoichiometry of thiophosphorylation was determined in each case (see Experimental Procedures) and found to be 1.0 thiophosphate/$\alpha_2\beta_2$ tetramer for the enzyme from incubations A–D inclusive and 0.07 thiophosphate/tetramer for the sample from E, consistent with virtually total release of thiophosphate from the enzyme when incubated with both CoA and succinate under these conditions.

enzyme on the left half is shown to be concerted with phosphate transfer from enzyme to succinate on the right, although the data by no means exclude possibility that transfer on the right precedes that on the left. In that case it would be ATP binding on the left, instead of phosphoryl transfer, that would promote the reciprocal structural transition. Our data do clearly indicate, however, that even transient bisphosphorylation (interconversion of forms A and D) does not occur, since this would be expected to allow positional isotope exchange when E–PSO_2 was incubated with [$^{18}O_4$]ATP. Similarly, transient bisphosphorylation via interconversion of forms B and E is unlikely, since our data indicate that positional isotope exchange takes place only after –PSO_2 has been removed from the enzyme. Thus, we propose that the half-sites reactivity of this enzyme persists to the level of transient intermediates in catalysis and that the catalytically driven transitions preserve structural asymmetry and contribute to catalytic efficacy.

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